Environmental Health Perspectives
Vol. 24, pp. 65–71, 1978

In Vitro Fertilization: A Potential Means for Toxicity Testing

by Benjamin G. Brackett*

Uses and potential uses of *in vitro* fertilization are: (1) a research tool for investigating biochemistry of fertilization, (2) an assay for fertilizing ability, (3) a potentially useful clinical approach for certain cases of infertility, and (4) a potentially useful means for improving animal breeding. *In vitro* fertilization methodology is sufficiently advanced for gametes of several mammalian species, especially mouse, rabbit, and rat, for use in evaluating effects imposed by toxic agents of environmental or genetic origin. Alteration of the normal events of fertilization and/or embryonic development following transfer of *in vitro* fertilized embryos into surrogate dams can serve as end point(s) in applying this means for toxicity testing. *In vitro* fertilization of mouse and rat ova has been explored as an alternative to *in vivo* fertilization in male contraceptive development studies. Original observations on toxicity of abnormal O_2 concentrations for rabbit fertilization *in vitro* are reported here. Ova were fertilized under 0, 20, 48, and 95% O_2, but toxicity from the extreme conditions, 0 and 95% was apparent from comparison of proportions of fertilized ova reaching the 4-cell stage during the 24-hr incubation. Toxicity was further evidenced by inability of embryos fertilized under 0 and 95% O_2 tensions, in contrast to those fertilized under 20% O_2, to sustain normal gestation following their transfer into recipient does. Recent success in the fertilization of cow ova *in vitro* in this laboratory provides encouragement to develop a useful means for testing normalcy of gametes in this species. Such studies might lead to useful screening procedures for avoidance of human infertility resulting from hazardous environmental conditions.

Introduction

The fertilization process begins with sperm penetration through the cellular investments (cumulus oophorus, and corona radiata), followed by penetration through the zona pellucida into the perivitelline space of the ovum. The fertilizing sperm cell then penetrates rapidly into the vitellus, where the sperm head containing nuclear material undergoes enlargement; the ovum undergoes activation which includes breakdown of cortical granules and extrusion of the second polar body (completion of meiosis). The remaining female chromatin condenses into the female pronucleus and the sperm head further enlarges to form the male pronucleus. The pronuclei grow and come together centrally and ultimately the membranes of the pronuclei breakdown; the chromosomes become mixed as mitosis begins and the fertilization process ends with cleavage of the zygote to form a two-cell stage embryo. This process requires 10 to 36 hr in various mammals. Initial reports providing adequate documentation for accomplishment of *in vitro* fertilization were carried out with sperm recovered from the uterus of mated rabbit does and consisted of cytological proof (*1*) and the birth of young following transfer of resulting embryos into recipient does (*2*). The necessity for exposure of sperm to the female reproductive tract prior to achievement of the capacity to fertilize (sperm capacitation) was recognized only a few years earlier (*3–5*).

The purpose of this paper is to review briefly *in vitro* fertilization, with emphasis on its uses and potential uses and to illustrate with original data an application of this approach to study toxicity of abnormal oxygen concentrations.

Basic Considerations

The work cited above paved the way for development of physical and chemical conditions compatible with fertilization of recently ovulated rabbit ova by capacitated sperm recovered from uteri of mated does (*6*). A simple defined medium

* Department of Clinical Studies, School of Veterinary Medicine and Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174.

was found capable of supporting the early stages of the fertilization process. Outstanding features of this medium were the high bicarbonate content adequate to maintain a pH of 7.8 under an atmosphere containing 5% CO_2, glucose as an energy substrate, and crystalline bovine serum albumin which was added in a concentration comparable to protein contributed by inclusion of serum in previous work. The advantageous effect of the bicarbonate-containing medium at pH 7.8 was realized from observations that when measurable volumes of uterine fluid were recovered with sperm suspensions in previous experiments optimal *in vitro* fertilization results were obtained and the pH of the fertilization medium in these cases was near 7.8 (*7*). Furthermore the same pH was detected for estrus rabbit oviductal fluid (*8*). The facility to combine ova and sperm cells from the same sources after varying treatments enabled comparison of resulting proportions of ova fertilized *in vitro* (*9*). This approach has been used in many subsequent experiments to learn more about the fertilization process and also to learn more about sperm capacitation (*10*). In the rabbit, ova can be recovered from three sites for *in vitro* fertilization, the oviducts just after ovulation (*6, 11*), the ovarian follicles just prior to ovulation (*12, 13*), or from the ovarian surface just after ovulation but prior to pickup by the oviduct (*13–16*).

Capacitation represents a change in the sperm cell which is manifested by increased metabolic activity (*17–19*) and involves alteration of surface antigens which otherwise prevent sperm penetration of ova (*16, 20, 21*). Alteration of sperm surface

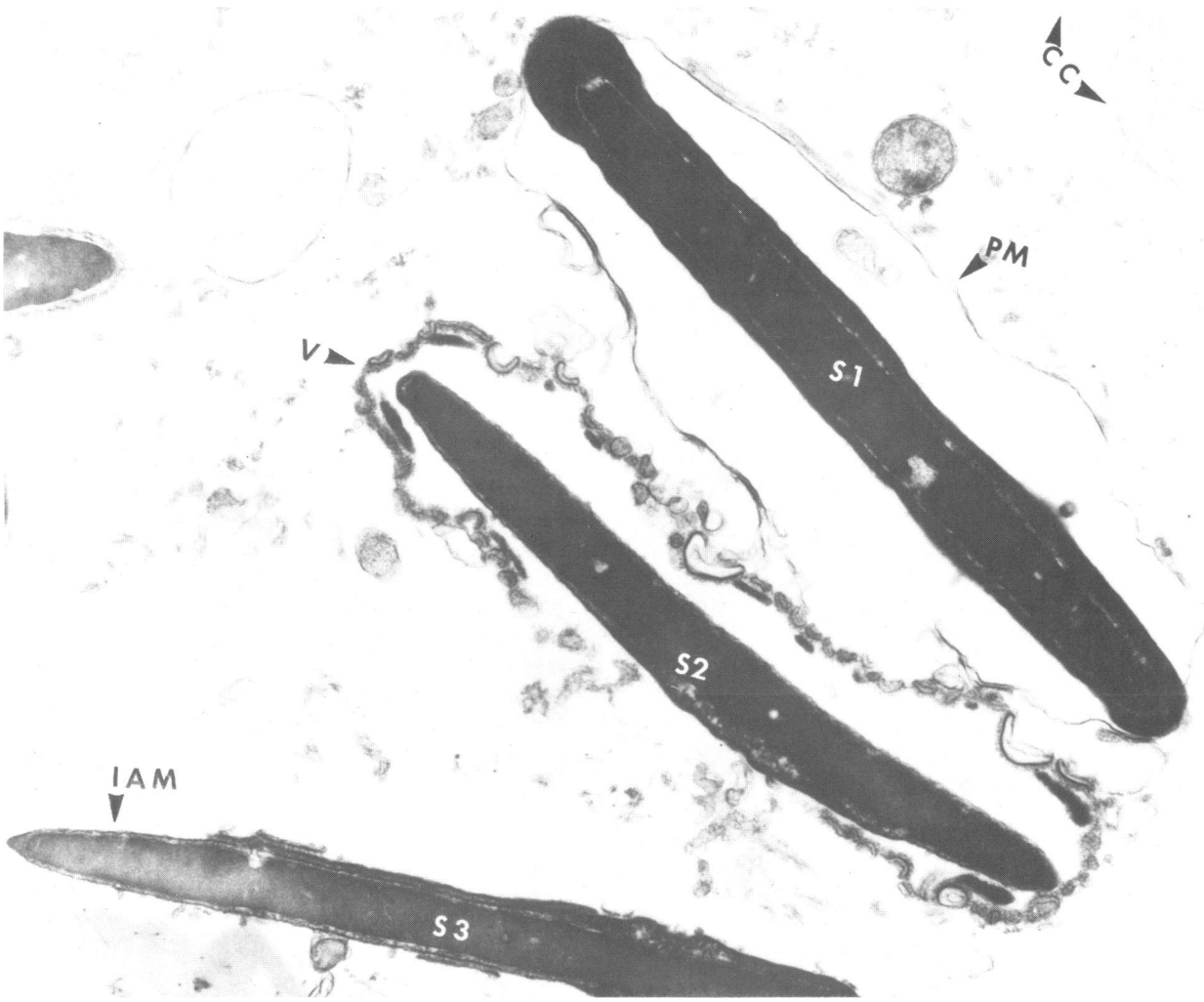

FIGURE 1. Rabbit sperm undergoing the acrosome reaction in the interstices between corona cells (CC) near the zona pellucida 1.5 hr after *in vitro* insemination (*25*). In sperm 1 (S1), the plasma membrane (PM) is swollen. In sperm 2 (S2) the plasma membrane and outer acrosomal membrane are undergoing vesiculation (V). In sperm 3 (S3), the acrosome reaction has been completed, exposing the inner acrosomal membrane (IAM).

coating antigenic components of seminal plasma by high ionic strength treatment enabled ejaculated rabbit sperm to fertilize ova *in vitro* (*16*). In addition to a possible ionic effect of oviductal fluid, the physiological mechanism may involve amylaselike enzyme activity within the female reproductive tract (*22*).

The sperm cell normally becomes conditioned within the female reproductive tract, i.e., capacitated, to undergo a morphological change, the acrosome reaction, upon contact with the ovum or its cellular investments (*23*). The acrosome reaction involves vesiculation which results from fusion of the plasma membrane with the outer acrosomal membrane ultimately leading to erosion with the inner acrosomal membrane exposed (*24*). Thin sections of three sperm heads in successive stages of the acrosome reaction, as seen in the electron microscope, are shown in Figure 1 (*25*). These events enable sperm enzymes responsible for entry into the ovum to become effective. Among the enzymes identified to play a role in sperm penetration hyaluronidase digests the matrix of the cumulus cell mass, the corona penetrating enzyme enables the sperm to reach the zona pellucida where the acrosomal proteinase, acrosin, enables digestion of the zona pellucida (*26*).

Following penetration through the zona pellucida, the fertilizing sperm cell rapidly contacts the vitellus and is assisted by microvilli of the ovum in penetration of the ooplasm. In most mammals, the rabbit being an exception, only one sperm cell penetrates through the zona pellucida. In all mammalian species normal fertilization involves penetration of only one sperm cell into the vitellus. Polyspermy denotes the situation in which more than one sperm cell penetrates the ovum. The abnormal condition of polyploidy may result from extra male pronuclei after polyspermy or from more than one female pronuclus, e.g., as follows suppression of meiosis and second polar body extrusion. In the latter case, triploidy results and when experimentally caused by appropriately timed colchicine treatment development of triploid embryos progresses to implantation but not to completion of gestation following transfer of experimental embryos (*27*). A variety of parthenogenetic and/or degenerative types of activation are recognized in the

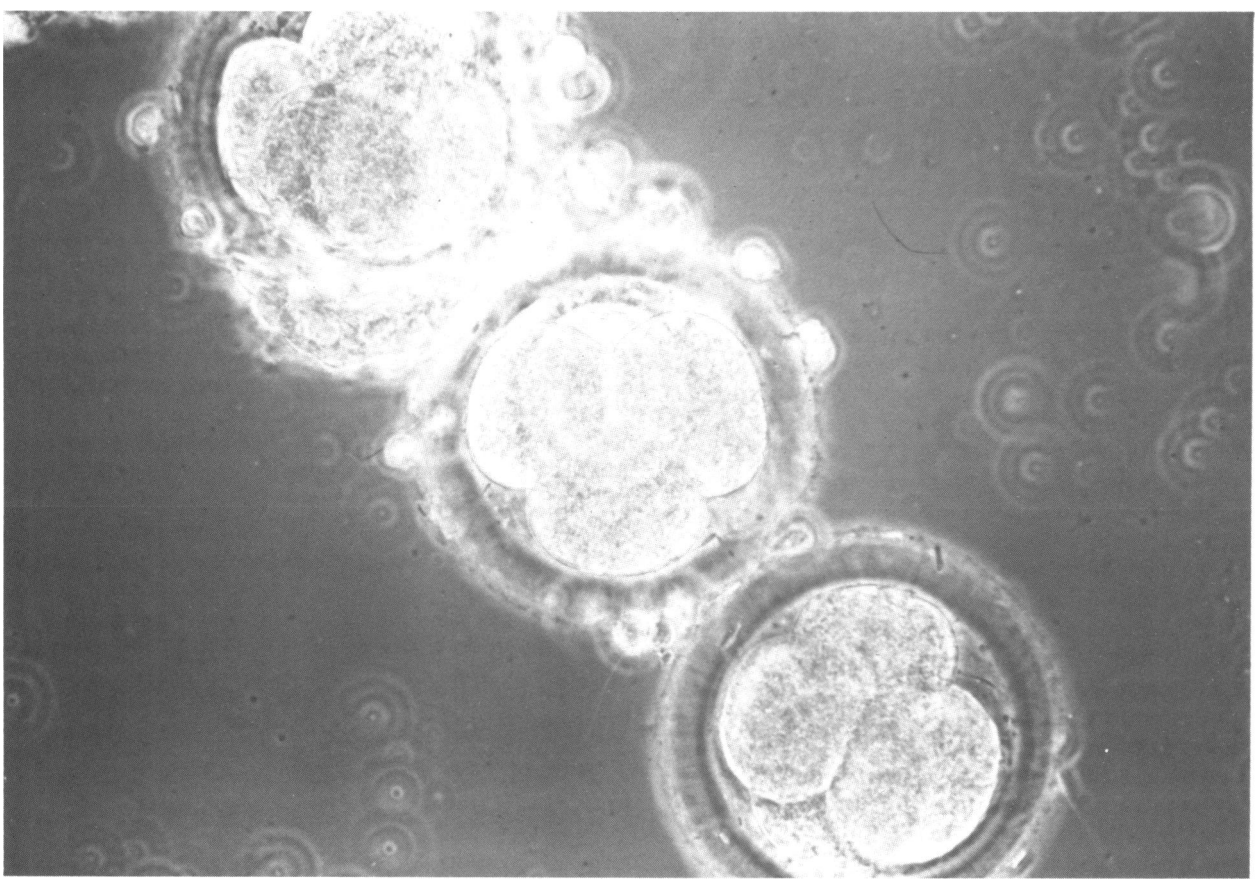

FIGURE 2. Normal-appearing four-cell stage rabbit embryos approximately 24 hr after *in vitro* insemination with rabbit sperm capacitated under optimal *in vivo* conditions.

mouse and other mammals, at least theoretically, but the potential for complete gestational development of such "embryos" is nil (*28, 29*).

Although 60 to 80% of the recipients of normal-appearing four-cell stage *in vitro* fertilized rabbit embryos, as shown in Figure 2, become pregnant, the proportions of recipients that deliver offspring are much lower, especially following *in vitro* incubation of the ova (*13*). Embryonic wastage, pre- and post-implantation, is great. Delivery of around 20% of the total number of *in vitro* fertilized rabbit embryos transferred was reported from this laboratory (*13*). Successful embryo transfer results have also followed fertilization and early embryonic development *in vitro* for the mouse (*30–36*) and rat (*37*).

Uses and Potential Uses of *in vitro* Fertilization

Uses and potential uses of *in vitro* fertilization come under four broad headings: (1) Research, e.g., to examine discrete events of the fertilization process; (2) assessment of fertilizing ability of egg and/or sperm after various treatments of gamete donor(s) or gamete(s); (3) to overcome infertility caused by blocked oviducts in women or oligospermia in men and stallions, or—coupled with embryo transfer—by any pathological conditions of oviduct and/or uterus in animals; (4) in animal production—coupled with embryo storage and transfer—to combine selected eggs with selected sperm for production of individual(s) with predicted characteristics at an appropriate time. The applicability of *in vitro* fertilization methodology has greatly facilitated progress in our understanding of fertilization and gamete physiology from investigational efforts during the last decade. Methodology is adequately described for several species to enable collection of meaningful data on ability of gametes to undergo fertilization, and with appropriate controls this approach can already be applied to discern effects of various forms of toxicity. Clinical applications are still best considered as potential uses of *in vitro* fertilization since success has not followed the best documented efforts to overcome carefully selected cases of human infertility by this approach. Nevertheless the clinical applications especially in the veterinary medical field, e.g. to overcome certain types of infertility, to better understand infertility, and to facilitate production of desirable animals, represent very attractive goals.

That *in vitro* fertilization methodology can be applied in testing for toxicity affecting the male reproductive tract has been demonstrated by Tsunoda and Chang (*38*) in work related to male contraceptive development. α-chlorohydrin was administered to male rats and mice, and the fertilizing ability of epididymal sperm from the treated males was tested for fertilizing ability *in vitro* and *in vivo* by intrauterine insemination. Fertilizing ability was reported as percentage of ova fertilized. In the mouse, the effective dose for inhibition of fertilization *in vivo* was higher than that required to produce the same effect *in vitro*, and infertility caused by the drug in the rat was found to be of shorter duration by *in vivo* testing than by *in vitro* testing. It was concluded that the *in vivo* approach for testing fertilizing ability of sperm from α-chlorohydrin-treated males was less laborious and preferable since it was more physiological and was more sensitive, at least for the rat. Given a ready source of oocytes for study in an animal with a long gestation period, as in nonhuman primates or the cow, if similar results could follow *in vitro* fertilization as those attainable through *in vivo* testing the desired approach would undoubtedly be that of *in vitro* fertilization.

A clue to genetic toxicity might follow observations of abnormal fertilization as reported following *in vivo* studies in mice (*39*). In a recent study tertiary trisomic male mice, carrying the small translocation chromosome from the T(1;13)7OH recipocal mouse translocation as the extra chromosome, were oligospermic, and this condition led to delayed fertilization. It was concluded that there was no relationship between sperm morphology and karyotype. An earlier study involving sperm distribution in the female reproductive tract following matings with sterile t^{w18}/t^{w32} male mice revealed normal sperm numbers in the uterus but few or no sperm at the site of fertilization in the oviducts (*40*). Abnormal sperm morphology could not account for these findings. In Friesian bulls, abnormal development of acrosomes resulting in so-called "knobbed" sperm was implicated in sterility resulting from an autosomal sex-limited recessive gene influenced by local environmental conditions within the testes (*41*). A greater proportion of abnormal embryos might result from insemination *in vitro* than *in vivo* under conditions involving genetic aberrations and following cytogenetic evaluation insights to mechanisms of genetic toxicity, i.e., infertility, might be forthcoming.

Experimental Observations on Fertilization of Rabbit Ova under Various Oxygen Tensions

In general, internal fertilization of mammalian species is well protected from environmental toxicity in the normal sense as it might concern other

organ systems in which a more direct effect is seen, e.g., respiratory. However, adverse influences might also alter the environment in which fertilization or events before or after fertilization take place. Experiments reported here illustrate effects that might be observed as a result of extreme alterations in the normal milieu for fertilization and very early embryonic development.

Materials and Methods

Ova were recovered from the ovarian surfaces of superovulated Dutch Belted and New Zealand White donors as previously described (*14*), and sperm were recovered from uterine horns of mated does (capacitators). Each capacitator was mated by two to four bucks 18 hr earlier. The medium was a simple defined medium routinely adopted for rabbit *in vitro* fertilization in this laboratory (*16*); in these experiments paraffin oil was used to completely fill the space above the 4.0 ml gamete-containing solution for incubation in small (30 mm diameter, 12 mm deep) tissue culture dishes.

In each experiment, desired combinations of O_2 and N_2 along with 5% CO_2 were mixed by flowmeters and used for equilibration of the medium and paraffin oil and for maintaining the same atmosphere above the incubation dish throughout the 24-hr incubation interval. Rapid equilibration of the gas phase was achieved following *in vitro* insemination by use of a small chamber (anaerobic culture apparatus, small size, Arthur H. Thomas Co., Philadelphia, PA).

Approximately 24 hr after *in vitro* insemination, ova were examined for evidence of fertilization, i.e., normal-appearing cleavage stages with chromatin demonstrable within each blastomere of representative ova (*12*). Proportions of ova that were fertilized under various conditions were compared statistically by chi-square analysis. Embryo transfers were carried out as previously described (*13*) to assess the potential for *in vivo* development.

Results

Results of 15 experiments are summarized in Table 1. The percentages of ova fertilized at various oxygen tensions *in vitro* did not vary significantly, suggesting that once the gametes are prepared for fertilization, the process of gamete union can procede even under adverse conditions. However, upon comparison of proportions of fertilized ova developing to at least the four-cell stage within the incubation interval highly significant differences were apparent. A significantly greater proportion of ova fertilized and incubated under an atmosphere of 20% oxygen developed to at least the four-cell stage than was the case under atmospheres of 0% oxygen ($p < 0.05$) or 95% oxygen ($p < 0.05$). Development of ova under 48% oxygen was similar to that seen under 20% oxygen in this work.

Table 1. Oxygen tension and fertilization of rabbit ova *in vitro*.

O_2, %[a]	Ratio ova fertilized / ova inseminated	% fertilized	Development during 24 hr: No. 2-cell stage	Development during 24 hr: No. at least 4-cell stage
0	24/38	63	7	17
20	114/163	77	6	108
48	8/10	80	1	7
95	19/47	41	16	3

[a] Gas phases (% by volume) for equilibration of medium, paraffin oil, and atmosphere contained 5% CO_2, O_2 as indicated, and balance N_2.

The effect of hostile conditions resulting from inappropriate levels of oxygen tension were also reflected in results of embryo transfers. Two experiments were done (using ova and sperm from the same sources) in which the viability of embryos resulting from fertilization under 20% oxygen was compared with that of embryos developing under 0% and 95% oxygen tensions, respectively. In the first, seven embryos (two in eight-cell and five in four-cell stages) that were fertilized under 20% oxygen were transferred to the left oviduct and seven embryos (four-cell stage) that were fertilized under 0% oxygen were transferred to the right oviduct of the same hormonally synchronous recipient doe; four embryos from the 20% oxygen group implanted and continued their development into normal offspring, while one of the embryos from the oxygen-deficient group implanted but did not survive to term. In the second embryo transfer experiment, five embryos (four-cell stage) that were fertilized under 20% oxygen were transferred into the right oviduct, and six embryos (two-cell stage) that were fertilized under 95% oxygen were transferred into the left oviduct of the same recipient; one of the embryos from the 20% oxygen group was resorbed after implantation, but two others developed into normal offspring while none of the embryos from the 95% oxygen group implanted.

Discussion of Experimental Observations and Concluding Remarks

In previously reported experiments fertilization of 61.8% (34/55) of tubal ova followed sperm penetration with measured oxygen tensions of 2.49 to 3.27 μl O_2/ml, or approximately 9 to 12% O_2 (*9*). In recent work, equilibration of medium, covering oil,

and atmosphere with 5% CO_2, 8% O_2, and balance N_2 has been adopted, since this oxygen tension is comparable to that reported in the oviduct (*42, 43*). Fertilization results over a wide range of oxygen tensions were similar and only under extreme conditions did toxic effects become recognizable (Table 1). This also appears to be the case for *in vitro* culture of mouse embryos (*44*). Whitten (*45*) found improvement of *in vitro* mouse embryo development under conditions with oxygen levels more comparable to those *in vivo*, i.e., 5% O_2. Baeckeland (*46*) used Whitten's conditions and observed development of mouse embryos from the two-cell stage to blastocysts, but these conditions did not support similar development of rat embryos. Haidri, Miller and Gwatkin (*47*) reported best *in vitro* maturation of mouse oocytes under 5–10% O_2, while in later work a well-defined optimum oxygen concentration was reported to be 5% (*48*).

Although results following experimentally induced oxygen deprivation and oxygen toxicity do not correspond to any recognized environmental condition, aside from death in the case of 0% O_2 tension (*43*), these experiments serve to illustrate the use of *in vitro* fertilization for toxicity testing.

Although drastic conditions were required to interfere with reproduction in the original experiments reported here, less drastic conditions might over a period of time result in pathological conditions of the gonads and hence, defective gametes. For the future, insight regarding abnormal fertilization resulting from environmental (or genetic) insult might follow *in vitro* fertilization studies in the cow and other grazing animals. The exposure of domestic animals to environmental pollutants near major urban areas might be reflected in performance of their gametes. Some progress has already been made in development of procedures for *in vitro* fertilization in the pig (*49*) and in the cow (*50, 51*). In this laboratory cow ova recovered near the time of ovulation have been fertilized by *in vitro* capacitated sperm and cleavage observed to the four-cell stage. Future detection of gonadal toxicity might become apparent in an ancillary way through *in vitro* fertilization primarily designed for evaluating the breeding performance of individual bulls or cows. Although many of the thoughts presented here are highly speculative today, *in vitro* fertilization can realistically be considered a potential means for toxicity testing.

The author gratefully acknowledges support of Grant HD 09406 from NICHHD.

REFERENCES

1. Thibault, C., Dauzier, L., and Wintenberger, S. Etude cytologique de la fecondation *in vitro* de l'oeuf de la lapine. C. R. Soc. Biol. (Paris) 148: 789 (1954).
2. Chang, M. C. Fertilization of rabbit ova *in vitro*. Nature 184: 466 (1959).
3. Austin, C. R. Observations on the penetration of sperm into the mammalian egg. Austral. J. Biol. Sci., B4: 481 (1951).
4. Austin, C. R. The capacitation of the mammalian sperm. Nature 179: 326 (1952).
5. Chang, M. C. Fertilizing capacity of spermatozoa deposited in the fallopian tubes. Nature 168: 697 (1951).
6. Brackett, B. G., and Williams, W. L. Fertilization of rabbit ova in a defined medium. Fertil. Steril. 19: 144 (1968).
7. Brackett, B. G., and Williams, W. L. *In vitro* fertilization of rabbit ova. J. Exptl. Zool. 160: 271 (1965).
8. Bishop, D. W. Metabolic conditions within the oviduct of the rabbit. Int. J. Fertil. 2: 11 (1957).
9. Brackett, B. G. Effects of washing the gametes on fertilization *in vitro*. Fertil. Steril. 20: 127 (1969).
10. Brackett, B. G. Extracorporeal fertilization of mammalian ova. In: Gynecologic Endocrinology, 2nd ed., J. J. Gold, Ed. Harper and Row, New York, 1975.
11. Brackett, B. G. *In vitro* fertilization of rabbit ova: time sequence of events. Fertil. Steril. 21: 169 (1970).
12. Brackett, B. G., Mills, J. A., and Jeitles, G. G. *In vitro* fertilization of rabbit ova recovered from ovarian follicles. Fertil. Steril. 23: 898 (1972).
13. Mills, J. A., Jeitles, G. G., and Brackett, B. G. Embryo transfer following *in vitro* and *in vivo* fertilization of rabbit ova. Fertil. Steril. 24: 602 (1973).
14. Brackett, B. G., and Server, J. B. Capacitation of rabbit spermatozoa in the uterus. Fertil. Steril. 21: 687 (1970).
15. Seitz, H. M., Jr., Brackett, B. G., and Matroianni, L., Jr. *In vitro* fertilization of ovulated rabbit ova recovered from the ovary. Biol. Reprod. 2: 262 (1970).
16. Brackett, B. G., and Oliphant, G. Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12: 260 (1975).
17. Hamner, C. E., and Williams, W. L. Effect of the female reproductive tract on sperm metabolism in the rabbit and fowl. J. Reprod. Fert. 5: 143 (1963).
18. Mounib, M. S., and Chang, M. C. Effect of *in utero* incubation on the metabolism of rabbit spermatozoa. Nature 201: 943 (1964).
19. Brackett, B. G. Respiration of spermatozoa after *in utero* incubation in estrus and pseudopregnant rabbits. Intern. Cong. Anim. Reprod. A. I. (Paris) 6th Congr. 1: 43 (1968).
20. Oliphant, G., and Brackett, B. G. Immunological assessment of surface changes of rabbit sperm undergoing capacitation. Biol. Reprod. 9: 404 (1973).
21. Reyes, A., Oliphant, G., and Brackett, B. G. Partial purification and identification of a reversible decapacitation factor from rabbit seminal plasma. Fertil. Steril. 26: 148 (1975).
22. Hall, J. L., and Brackett, B. G Enzymatic capacitation of ejaculated rabbit sperm *in vitro*. Paper presented at Society for the Study of Reproduction Meeting, 1976, Philadelphia, Abstr. No. 10, 20.
23. Bedford, J. M. Ultrastructural changes in the sperm head during fertilization in the rabbit. Am. J. Anat. 123: 329 (1968).
24. Barros, C., et al. Membrane vesiculation as a feature of the mammalian acrosome reaction. J. Cell Biol. 34: C1 (1967).
25. Oh, Y. K., and Brackett, B. G. Ultrastructure of rabbit ova recovered from ovarian follicles and inseminated *in vitro*. Fertil. Steril. 26: 665 (1975).
26. McRorie, R. A., and Williams, W. L. Biochemistry of mammalian fertilization. In: Annual Review of Biochemis-

try, Vol. 43, E. E. Snell, Ed., Annual Reviews, Palo Alto, 1974.
27. Bomsel-Helmreich, O. Heteroploidy and embryonic death. In: Ciba Symposium on Pre-implantation Stages of Pregnancy. G. E. W. Wolstemholme and M. O'Connor, Eds. Churchill, London, 1965, p. 246.
28. Graham, C. F., and Duessen, Z. A. *In vitro* activation of mouse eggs. J. Embryol. Exptl. Morph. 31: 497 (1974).
29. Kaufman, M. H., and Surani, M. A. H. The effect of osmolarity on mouse parthenogenesis. J. Embryol. Exptl. Morph. 31: 513 (1974).
30. Cross, P. C., and Brinster, R. L. *In vitro* development of mouse oocytes. Biol. Reprod. 3: 298 (1970).
31. Hoppe, P. C., and Pitts, S. Fertilization *in vitro* and development of mouse ova. Biol. Reprod. 8: 420 (1973).
32. Kaufman, M. H., and Whittingham, D. G. Viability of mouse oocytes ovulated within 13 hours of an injection of pregnant mares serum gonadotropin. J. Reprod. Fertil. 28: 465 (1972).
33. Miyamoto, H., and Chang, M. C. Development of mouse eggs fertilized *in vitro* by epididymal spermatozoa. J. Reprod. Fertil. 30: 135 (1972).
34. Mukherjee, A. B. Normal progeny from fertilization *in vitro* of mouse oocytes matured in culture and spermatozoa capacitated *in vitro*. Nature 237: 397 (1972).
35. Mukherjee, A. B., and Cohen, N. M. Development of normal mice by *in vitro* fertilization. Nature 228: 472 (1970).
36. Whittingham, D. G. Fertilization of mouse eggs *in vitro*. Nature 220: 592 (1968).
37. Toyoda, Y., and Chang, M. C. Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fert. 36: 9 (1974).
38. Tsunoda, Y., and Chang, M. C. Fertilizing ability *in vivo* and *in vitro* of spermatozoa of rats and mice treated with alpha-chlorohydrin. J. Reprod. Fert. 46: 401 (1976).
39. de Boer, P., van der Hoeven, F. A., and Chardon, J. A. P. The production, morphology, karyotypes and transport of spermatozoa from tertiary trisomic mice and the consequences for egg fertilization. J. Reprod. Fert. 48: 249 (1976).
40. Olds, P. J. Effect of the T locus on sperm distribution in the house mouse. Biol. Reprod. 2: 91 (1970).
41. Donald, H. P., and Hancock, J. L. Evidence of gene controlled sterility in bulls. J. Agric. Sci. 43: 178 (1953).
42. Bishop, D. W. Oxygen concentrations in the rabbit genital tract. Int. Congr. Anim. Reprod. 3: 53 (1956).
43. Mastroianni, L., Jr., and Jones, R. Oxygen tension within the rabbit fallopian tube. J. Reprod. Fert. 9: 99 (1965).
44. Auerbach, S., and Brinster, R. L. Effect of oxygen concentration on the development of two-cell mouse embryos. Nature 217: 465 (1968).
45. Whitten, W. K. Nutrient requirements for the culture of preimplantation embryos. In: Schering Symposium of Intrinsic and Extrinsic Factors in Early Mammalian Development. (Adv. Biosci. 6) p. 12. G. Raspe, Ed., Pergamon Press, Oxford, 1971.
46. Baeckeland, E. Influence de la teneur en oxygène de la phase gazeuse sur la culture d'oeufs de souris et de rat. C. R. Soc. Biol. (Paris) 169: 449 (1975).
47. Haidri, A. A., Miller, I. M., and Gwatkin, R. B.L. Culture of mouse oocytes *in vitro*, using a system without oil or protein. J. Reprod. Fert. 26: 409 (1971).
48. Gwatkin, R. B. L., and Haidri, A. A. Oxygen requirements for the maturation of hamster oocytes. J. Reprod. Fert. 37: 127 (1974).
49. Iritani, A., Sato, E., and Nishikawa, Y. Fertilization of pig oocytes *in vitro* with capacitated spermatozoa. Paper presented at Society for the Study of Reproduction Meeting, 1974; Abstracts 111: 115 (1974).
50. Iritani, A., and Niwa, K. Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. J. Reprod. Fert. 50: 119 (1977).
51. Brackett, B. G., et al. Bovine fertilization and early development *in vivo* and *in vitro*. Paper presented at Society for the Study of Reproduction Meeting, Austin, Texas, 1977. Abstr. 86: 56 (1977).